In the claims:

Please amend the claims as follows:

1. A polymer-modified monovalent antibody fragment [compromising] comprising an antigen-binding fragment and at least one polymer molecule in covalent linkage characterised in that each cysteine residue located in the antigen-binding fragment outside of the variable region domain is either covalently linked through its sulphur atom to a polymer molecule or is in disulfide linkage with a second cysteine residue located in the antigen-binding fragment;

wherein said antigen-binding fragment is covalently linked to one, two or three polymer molecules through one, two or three cysteine residues located in the antigen-binding fragment outside its variable domain;

and wherein said polymer is [an] optionally substituted, and is a straight or branched chain polymer selected from the group consisting of poly(ethylene glycol), [poly(ethylene glycol),] poly(propylene glycol), poly(vinyl alcohol) and derivatives [thereof] of poly(ethylene glycol), poly(propylene glycol) and poly(vinyl alcohol).

Remarks

I. Rejections under 35 USC § 112, Second Paragraph

Claims 1, 5, 9-11 have been rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim what the applicant regards as the invention.

Claims 1, 9-11 are allegedly indefinite in the recitation of "derivatives thereof" in claim 1. Claim 1 has been amended to recite that the derivatives are "of poly(ethylene glycol), poly(propylene glycol), and poly(vinyl alcohol)." The term "derivatives" is defined on page 6, lines 26-32 of the specification as to mean reactive derivatives, for example thiol-selective reactive groups such as maleimides and the like. This rejection has be obviated by amendment.

Claims 1, 5, 9-11 are allegedly indefinite for reciting "optionally" in claim 1. Claim 1 has been amended to recite "wherein said polymer is optionally substituted, and is a straight or branched chain polymer..." The phrase "optionally substituted" in the amended claim 1 clearly modifies "said polymer." The phrase "optionally substituted" is defined in the specification as including "one or more hydroxy, methyl or methoxy groups." (Specification, page 6, lines 18-20.) This rejection has been obviated by amendment.

Claim 1 is allegedly indefinite because it is allegedly structured as an improper Markush claim. In particular, the Examiner contends that it is not clear what the phrase "optionally substituted" is intended to modify. Claim 1 has been amended to recite "wherein said polymer is optionally substituted, and is a straight or branched chain polymer..." The phrase "optionally substituted" in the amended claim 1 clearly modifies "said polymer." This rejection has been obviated by amendment.

In view of the foregoing, Applicants respectfully request that the rejections under 35 U.S.C. § 112, second paragraph be withdrawn.

II. Rejections under 35 U.S.C. § 102(b)

Claims 1 and 9-10 remain rejected, and claim 11 is newly rejected under 35 U.S.C. § 102 (b) as allegedly anticipated by Pedley et al. (Br. J. Cancer 70:1126-30, 1994). Applicants traverse this rejection. Pedley et al. do not teach each and every element of the invention.

The Examiner asserts that Pedley et al. teaches "site specific modification of cysteine residues", yet the Examiner only provides a general citation where this teaching can be found -- i.e., page 1127, left column. (Official Action of July 20, 2001, page 4, lines 3-5.) Applicants respectfully request a more specific citation to this alleged teaching, either by line number or paragraph. Pedley et al. does not teach site specific modification of cysteine residues, but rather teach **random** PEG attachment via **lysine** residues. (See specification as filed, page 3, line 1-7.) Pedley et al. teach a maleimide-containing derivative of PEG conjugated to thiols that are introduced to the protein at lysine residues by the use of 2-iminothiolane (Traut's reagent). (Pedley et al., page 1127, left column, lines 2-5) Because these thiol groups are introduced to the protein by 2-iminothiolane, they are not confined to naturally occurring thiols, such as those found on cysteines residues, but may be found on any residue originally having an amino group. In fact, 2-iminothiolane acts primarily on lysines. (Kenny, J.W., et al., Methods in Enzymology, 1979, 59:534-550, page 534, bottom, enclosed herewith.) Regardless, it is unlikely that PEG is conjugated to a cysteine in the Fab' fragment of Pedley et al. because, prior to the PEG conjugation, the "liberated hinge thiols" of the Fab' were alkylated and therefore were unavailable for PEG-modification. (Pedley et al., page

126, right column, lines 33-34.) Therefore the PEG molecules of the modified Fab' fragments of Pedley et al. are not found solely, if at all, on cysteine residues, and Pedley et al. do not teach every element of the claimed invention.

The Examiner asserts that cysteine residue of claim 1 would encompass any cysteine residue, "even those not found naturally in the antibody." (Official Action of July 20, 2000, page 4, line 16.) If the Examiner would like to assert that non-native cysteines would be formed by the treatment of a protein with 2-iminothiolane, Applicants request that he provide a reference or file a declaration pursuant to 37 C.F.R. § 1.104(d)(2) to support his position.

Pedley et al. do not teach a monovalent antibody fragment "where each cysteine residue outside of the variable region domains is either covalently linked through its sulfur atom to a polymer molecule or is in disulfide linkage with a second cysteine residue located in the antigen-binding fragment." As stated above, the hinge thiols, which are from cysteine residues, are alkylated prior to PEG conjugation. Therefore, in Pedley et al. each cysteine residue is **not** linked to a polymer molecule or to a second cysteine residue, and Pedley et al. do not teach every element of the invention.

In view of the foregoing, Applicants respectfully request that the rejection under 35 U.S.C. § 102(b) be withdrawn

III. Rejections under 35 U.S.C. § 102(e)

Claims 1 and 9-11 have been rejected under 35 U.S.C. § 102(e) as being allegedly anticipated by Griffiths et al. (U.S. Patent 5,670,132). Griffiths et al. allegedly teach site specific attachment of PEG to thiols in an antigen binding fragment outside the variable region, the antigen binding fragment being a Fab or Fab', and having an effector attached. Applicants traverse this rejection. Griffiths et al. does not disclose every element of the invention and does not enable one of ordinary skill in the art to make the invention.

Griffiths et al. does not teach a polymer modified monovalent antibody fragment where "**each** cysteine residue located in the antigen-binding fragment outside the variable region domain is either covalently linked through its sulfur atom to a polymer molecule or is in disulfide linkage with a second cysteine residue located in the antigen-binding fragment." (emphasis supplied.) Griffiths et al. teach that in the useful end-product contemplated, at least some of the cysteine thiol groups are attached to Tc-99m. (Griffiths et al., column 3, lines 62-64.) Each of the cysteines of the polymer-modified antibody fragments of Griffiths et al. is not either bound to a second cysteine or to a polymer molecule, as recited in the claims. Therefore, Griffiths et al. does not disclose each of the elements of the claimed invention.

Further, Griffiths et al. does not provide the degree of disclosure necessary for one skilled in the art to make the monovalent antibody fragments of the claimed invention. Griffiths et al. teach that "the intact immuno-globulin is first partially reduced under mild conditions to produce free thiol groups, which are then reacted with a PEG derivative capable of selective reaction with thiols." (Griffiths et al., column 3, line 66 - column 4, line 2.)

Further, "the PEG conjugate is then purified by size exclusion chromatography and proteolytically cleaved by standard methods with pepsin to produce $F(ab')_2$ fragments, or papain to produce $F(ab)_2$ [sic] fragments." (Griffiths et al., column 4, lines 8-12.) In order to proteolytically cleave the PEG conjugate with pepsin to yield bivalent $F(ab')_2$, the fragments, the disulfide bonds in the hinge region of the PEG conjugate must be intact. The "mild conditions" used to partially reduce the intact immuno-globulin in the first step must therefore leave the hinge disulfide bonds intact. The nature of the "mild conditions" critical to reduce thiols but not the more accessible hinge disulfide bonds is not taught in Griffiths et al. Further, in the discussion regarding the subsequent labelling with Tc-99m, Griffiths et al. acknowledges that the hinge region disulfide bonds are more accessible to reducing agents. (Griffiths et al., column 4, lines 55-58.) In the examples, however, Griffiths et al. teaches that the **lysines** are pegylated. (Griffiths et al., column 7, lines 55-61, emphasis supplied.) Griffiths et al, thus, do not disclose how to specifically modify cysteine residues with PEG.

Finally, Griffiths et al. is very confusing in its discussion of antibody fragments. It is unclear from the specification what is meant by a $F(ab)_2$ fragment. (Griffiths et al., column 3, line 1; column 4, line 11; column 5, line 4.) It is well known in the art that there are $F(ab')_2$ bivalent antibody fragments that retain a covalently bonded hinge region, and which may be reduced to monovalent F(ab') fragments. It is also well known that there are F(ab) monovalent antibody fragments which do not have the hinge regions. However, the identity of $F(ab)_2$ bivalent antibody fragments is not well known in the art. (See Janeway et al., ImmunoBiology, pp. 82-83, enclosed herewith.) Griffiths et al. is so inaccurate and

incomplete in its teachings of polymer-modified monovalent fragments, that one of skill in the art would not be able to use its teachings to make the invention of claims 1 and 9-11.

In view of the foregoing, Applicants respectfully request that the rejection under 35 U.S.C. § 102(e) be withdrawn.

IV. Rejections under 35 U.S.C. § 103(a)

Claims 1, 5 and 9-11 have been rejected under 35 U.S.C. 103(a) as being allegedly unpatentable over Pedley et al. (Br. J. Cancer 70:1126-30, 1994) in view of Goodson et al. (Bio/Technology 8:343-346, 1990) or Woghiren et al. (Bioconjugate Chem. 4:314-318, 1993). Pedley et al. allegedly teach the covalent attachment of poly(ethylene glycol) to an antibody, a Fab fragment and a Fab' fragment of anti CEA. Goodson et al. or Woghiren et al. allegedly teach a protein modified with the addition of methoxy(polyethylene glycol). Applicants traverse this rejection. As discussed above, discussion incorporated herein, Pedley et al. does not disclose or suggest Applicants' invention. The remaining references do not overcome the deficiencies of Pedley et al. Further, no motivation is provided in Pedley et al., Goodson et al. or Woghiren et al. to combine these references and make obvious the claimed invention.

From the teaching of Pedley et al., one of ordinary skill in the art would not be motivated to combine Pedley et al. with Goodson et al. or Woghiren et al. which the Examiner asserts teach the addition of methoxy(polyethylene glycol) to **cysteine** residues in

the protein. (Official Action, page 8, lines 13-14.) Regardless, even if combined, the references do not yield Applicants' invention.

Woghiren et al. do not suggest the advantage of using methoxy(polyethylene glycol) to modify antibody fragments. The Examiner asserts that Woghiren et al. provide motivation to combine, citing the following: "we have prepared an activated form of PEG that is a stable reagent, but readily reacts with the thiol group of cysteine to form a disulfide - linked PEG adduct ." (Official Action, page 9, lines 6-7.) However, the mere availability of an activated form of PEG that is a stable reagent gives no motivation to use the reagent specifically with antibodies, and more specifically with monovalent antibody fragments, and no expectation of success in this use. In fact, Woghiren et al. actually teaches away from the use of PEG to modify antibodies. Woghiren et al. cite a reference in which PEG-modification inactivated a monoclonal antibody. (Woghiren et al., Introduction, line 14.)

Claims 1, 5 and 9-11 have been rejected under 35 U.S.C. 103(a) as being allegedly unpatentable over Griffiths et al. (U.S. Patent 5,670,132) in view of Goodson et al. (Bio/Technology 8:343-346, 1990) or Woghiren et al. (Bioconjugate Chem. 4:314-318, 1993). Griffiths et al. allegedly teach the site specific attachment of PEG to thiols in an antigen binding fragment outside the variable region, *intra alia*. Goodson et al. or Woghiren et al. allegedly teach a protein modified with the addition of methoxy(polyethylene glycol). Applicants traverse this rejection. As discussed above, discussion incorporated herein, Griffiths et al. does not disclose or suggest Applicants' invention. The remaining references

do not overcome the deficiencies of Griffiths et al. Further, no motivation is provided in Griffiths et al., Goodson et al. or Woghiren et al. to combine these references and make obvious the claimed invention.

From the teaching of Griffiths et al., one of ordinary skill in the art would not be motivated to combine Griffiths et al. with Goodson et al. or Woghiren et al. which the Examiner asserts teach the addition of methoxy(polyethylene glycol) to cysteine residues in the protein. (Official Action, page 8, lines 13-14.) Regardless, even if combined, the references do not yield Applicants' invention.

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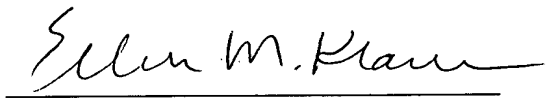
In view of the foregoing, Applicants respectfully request that the rejections under 35 U.S.C. § 103(a) be withdrawn.

For the foregoing reasons, Applicants submit that the present claims meet all the requirements for patentability. The Examiner is respectfully requested to allow all the present claims. If the Examiner is of a contrary view, he is requested to contact the undersigned at (215) 557-5948.

Respectfully submitted,



Ellen M. Klann, Ph.D.
Registration No. **44,836**

Date: 22 January 2001

Woodcock Washburn Kurtz
Mackiewicz and Norris LLP
One Liberty Place - 46th Floor,
Philadelphia, PA 19103,
(215) 557-5948

Attachments:

J.W. Kenny et al., Cross-linking of Ribosomes Using 2-iminothiolane (Methyl 4-Mercaptobutyrimidate) and Identification of Cross-Linked Proteins by Diagonal Polyacrylamide/Sodium dodecyl Sulfate Gel Electrophoresis. Methods in Enzymology, Vol. 59, pages 534-550.

C.A. Janeway et al., ImmunoBiology, 4th Edition, Elsevier Science Ltd., London/ Garland Publishing, New York, 1999, pages 82-83.

are lost. To examine the incorporation into these proteins, "three-dimensional" electrophoresis of acetic acid-soluble protein is available and is described in this volume [41].[18]

It must be added that these methods are useful for study of the metabolism of ribosomal proteins *in vivo*, as shown by our previous reports.[4-7]

[18] Y. Nabeshima, K. Imai, and K. Ogata, to be published.

[43] Cross-Linking of Ribosomes Using 2-Iminothiolane (Methyl 4-Mercaptobutyrimidate) and Identification of Cross-Linked Proteins by Diagonal Polyacrylamide/Sodium Dodecyl Sulfate Gel Electrophoresis[1]

By JAMES W. KENNY, JOHN M. LAMBERT, and ROBERT R. TRAUT

Many biological structures contain assemblies of different proteins. It is frequently valuable to determine the spatial relationships among the different protein components of the multiprotein complex. Bifunctional reagents have been used effectively to cross-link one protein component to others that occupy a suitably "neighboring" site in the structure or complex under investigation. A problem frequently encountered is that of identification of the monomeric components of cross-linked dimers or oligomers. The presence of a readily cleavable bond in the cross-linking reagent permits reversal of the cross-linking reaction and regeneration of monomeric components from isolated cross-linked complexes, thus facilitating their identification. Methods are described here that employ reversible cross-linking and analysis of a complex mixture of cross-linked products. They have been used successfully in the investigation of the protein topography of ribosomal subunits of *Escherichia coli*. They are of general applicability and are useful in the investigation of many other biological structures containing multiple protein components.

The reagent 2-iminothiolane, formerly called methyl 4-mercaptobutyrimidate[2,3] reacts with lysine amino groups in the intact ribosomal subunit to form amidine derivatives containing sulfhydryl groups. Disulfide bonds form when the modified subunit is subjected to oxidation.

[1] Supported by a research grant from the U.S. Public Health Service (GM 17924).
[2] R. R. Traut, A. Bollen, T. T. Sun, J. W. B. Hershey, J. Sundberg, and L. R. Pierce, *Biochemistry* 12, 3266 (1973).
[3] R. Jue, J. H. Lambert, L. R. Pierce and R. R. Traut, *Biochemistry*, in press (1978).

METHODS IN ENZYMOLOGY, VOL. LIX

ISBN 0-12-181959-0

Some of the disulfide bonds are intramolecular, while others are intermolecular and represent "cross-links" that provide information on the relative spatial arrangement of the different ribosomal proteins. The term "cross-link," when used in the remainder of the article, will imply intermolecular disulfide-linked proteins. It is a prerequisite for any cross-linking procedure that it not alter the structure under study. Various physical properties of ribosomal subunits, treated as described here, are not detectably altered. The cross-linked subunits retain the capacity to reassociate to form 70 S ribosomes and retain up to 50% of their activity in polyphenylalanine synthesis.[4]

Methods for the separation and identification of cross-linked dimers are described. Of particular general applicability is the technique of diagonal polyacrylamide/sodium dodecyl sulfate (SDS) gel electrophoresis.[5] It is a two-dimensional electrophoretic separation, utilizing the size dependence of the mobility of proteins in SDS to distinguish cross-linked from monomeric proteins. The first electrophoresis is performed under nonreducing conditions, and the second under reducing conditions. This results in a pattern in which non-cross-linked proteins fall on a diagonal line and cross-linked proteins fall beneath the diagonal.

Schematic diagrams of the modification and cross-linking reactions (Fig. 1) and of diagonal gel electrophoresis (Fig. 2) are shown. The procedures will be described in detail as they have been applied to the 50 S ribosomal subunit of *Escherichia coli*. In addition to the two general methods already mentioned, techniques for the purification of the mixture of cross-linked protein from 50 S ribosomal subunits prior to diagonal gel electrophoresis will be described.

Modification of 50 S Ribosomal Subunits with 2-Iminothiolane

Solutions

1. NH_4Cl, 100 m*M*; Tris·HCl, pH 7.2, 10 m*M*; $MgCl_2$, 10 m*M*; 2-mercaptoethanol, 14 m*M*
2. KCl, 50 m*M*; triethanolamine·HCl, pH 8.0, 50 m*M*; $MgCl_2$, 1 m*M*
3. Solution 2 with 5 m*M* dithiothreitol
4. 2-Iminothiolane, 500 m*M*; triethanolamine·HCl, pH 8.0, 500 m*M*; triethanolamine, free base, 500 m*M*

Tris was obtained from Sigma; 2-mercaptoethanol from BDH; dithiothreitol from Pierce; triethanolamine from Eastman and distilled

[4] J. M. Lambert, R. Jue, and R. R. Traut, *Biochemistry*, in press (1978).
[5] A. Sommer and R. R. Traut, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3946 (1974).

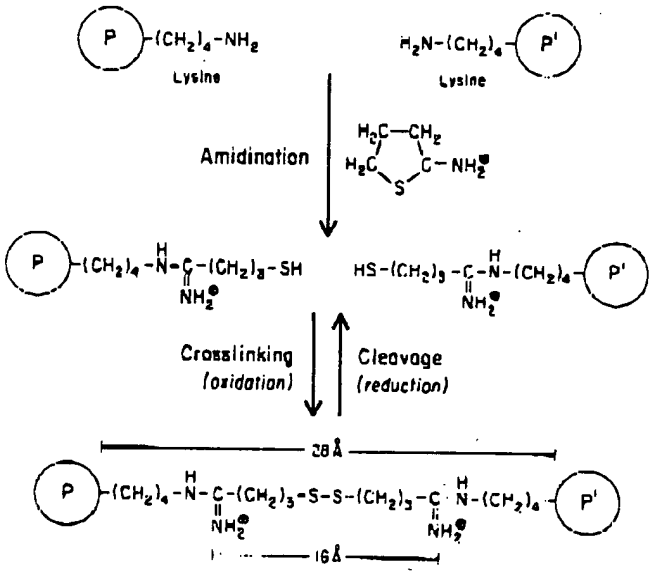


FIG. 1. Modification of proteins with 2-iminothiolane and reversible cross-linking by disulfide bond formation.

under vacuum prior to use. 2-Iminothiolane was prepared as described[5] or was purchased from Pierce. All reagents were of reagent grade.

Procedure

Radioactive 50 S ribosomal subunits were isolated from *E. coli* MRE600 grown in the presence of [^{35}S]sulfate as described[6] and were more than 95% free of contaminating 30 S subunits as determined by analytical centrifugation. They were stored in solution 1 at −70°. The specific radioactivity of the 50 S ribosomal protein was 170 × 10^6 cpm/mg.

The ribosomal subunits are reduced by incubation for 30 min at 30° in solution 1 to which 1% (v/v) 2-mercaptoethanol was added. The ribosomal subunits are then passed through a BioGel P-2 column (15 cm × 0.7 cm, for a 1.0-ml sample equilibrated with solution 3) in order to remove free amines that might react with 2-iminothiolane. The concentration of ribosomal subunits is adjusted to an A_{260} of 45 (1 mg of ribosomal protein per milliliter) with solution 3; 12 m*M* 2-iminothiolane (24 μl solution 4 per milliliter of sample) is added, and the mixture is incubated for 2.5 hr at 0°. The pH of the modification reaction is 8.0. Under these conditions each ribosomal protein reacts with on average two mol-

[5] T. T. Sun, A. Bollen, L. Kahan, and R. R. Traut, *Biochemistry* 13, 2334 (1974).

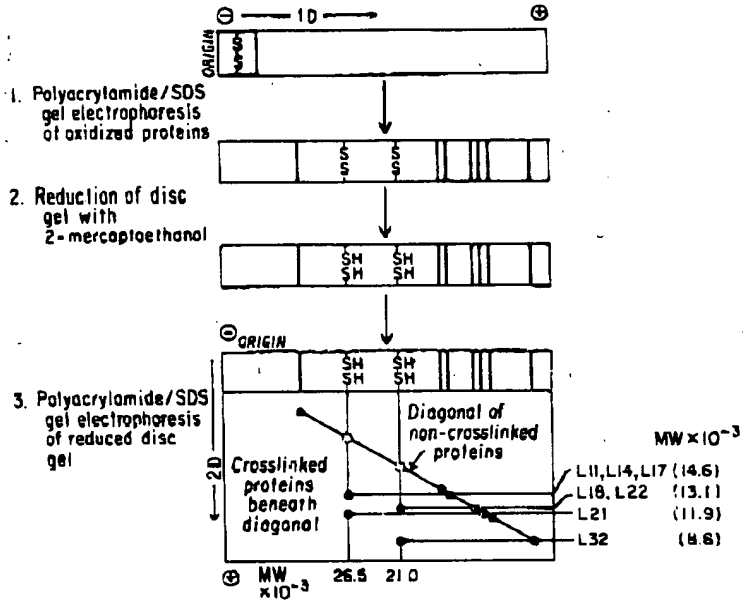


FIG. 2. Two-dimensional diagonal polyacrylamide/sodium dodecyl sulfate gel electrophoresis.

ecules of 2-iminothiolane.[3] The modified subunits are incubated with 40 m*M* hydrogen peroxide (4.5 μl of 30% hydrogen peroxide per milliliter of sample) at 0° for 30 min to promote cross-linking between adjacent sulfhydryl groups by disulfide bond formation. These reactions are represented in Fig. 1. Unreacted hydrogen peroxide is removed by the addition of catalase (15 μg of catalase per milliliter of sample) followed by incubation for 15 min at 0°. Unreacted 2-iminothiolane is removed either by passing the modified, oxidized sample through a BioGel P-2 column equilibrated with solution 2, or by dialysis against solution 2, in order to prevent reaction of the imidate with newly exposed amino groups in subsequent steps. Iodoacetamide is added to a concentration of 40 m*M*. The solution is incubated for 30 min at 30° to alkylate free sulfhydryl groups inaccessible to oxidation.

Extraction of Protein from Cross-Linked Ribosomal Subunits

Cross-linked 50 S ribosomal subunits are mixed with an equal volume of a solution containing 8 *M* urea (ultra pure), 6 *M* LiCl and 40 m*M* iodoacetamide (added immediately before use) and incubated at 0° for 24 hr. The precipitated RNA is removed by centrifugation at 10,000 rpm for 15 min. The supernatant protein fraction is dialyzed exhaustively against

6% acetic acid and lyophilized. Alternatively, the protein is precipitated by addition of 10 volumes of 10% (w/v) trichloroacetic acid and recovered by centrifugation at 10,000 rpm for 30 min at 4°. The precipitate is washed in ethanol/ether and dried under vacuum.

Two-Dimensional Diagonal Polyacrylamide/SDS Gel Electrophoresis

The disulfide bonds formed by oxidation of modified ribosomal subunits are readily cleavable by reduction. Polyacrylamide/SDS diagonal gel electrophoresis uses this property of the cross-link to separate intermolecular cross-linked dimers from protein monomers containing only intramolecular disulfide bonds. First the sample is electrophoresed under nonreducing conditions to maintain disulfide bonds intact. The proteins are reduced in the gel to cleave the disulfide bonds and convert cross-linked complexes into monomeric proteins. Monomeric proteins that had migrated as disulfide-linked complexes in the first dimension migrate more rapidly in the second electrophoresis. Uncross-linked proteins have the same electrophoretic mobility in both electrophoretic separations. The resulting protein pattern is composed of a diagonal line of non-cross-linked proteins with a complex array of cross-linked proteins below the diagonal. Figure 2 shows a schematic diagram of diagonal gel electrophoresis.

The SDS gel system described here gives a linear relationship between apparent log (molecular weight) and mobility for both cross-linked protein dimers and monomers between 10,000 and 60,000 daltons as calibrated using individual monomeric 30 S ribosomal proteins or commercially available molecular weight standards.[7] Within this range, the sum of the apparent molecular weights of the monomer proteins below the diagonal arising from a putative dimer is within 7.5% of that of the cross-linked complex. This additivity relationship together with the coincidence of the spots on the same vertical line provide the major criteria for identifying pairs of proteins originally cross-linked. The diagram in Fig. 2 shows these two criteria for assigning the monomeric proteins originating from a cross-linked dimer formed in the intact subunit.

Solutions and Acrylamide Gel Composition

5. SDS sample buffer, pH 6.8: SDS, 4% w/v; Tris·HCl, pH 6.8, 80 m*M*; iodoacetamide, 40 m*M*; glycerol, 10% v/v. The solution is

[7] A. Sumner and R. R. Traut, *J. Mol. Biol.* **97**, 471 (1975).

filtered and stored at room temperature. Iodoacetamide is added immediately before use.

6. Upper gel, pH 7.8: acrylamide, 5% w/v; *N,N'*-Methylenebisacrylamide (MBA), 0.26% w/v; SDS, 0.25% w/v; Tris·HCl, pH 7.8, 125 m*M*; tetramethylethylenediamine (TEMED), 0.05% v/v. The components are mixed and filtered at room temperature. The solution is degassed and polymerization is initiated by addition of 5ml per liter of a freshly prepared solution of ammonium persulfate (10% w/v).
7. Separation gel, pH 8.7: acrylamide, 17.5% w/v; MBA, 0.35% w/v; SDS, 0.1% w/v; Tris·HCl, pH 8.7, 335 m*M*; TEMED, 0.033% v/v. The components are mixed, filtered, and degassed. Polymerization is initiated by addition of 6.6 ml per liter of a freshly prepared solution of ammonium persulfate (10% w/v). The final acrylamide:bisacrylamide ratio is 30:0.6.
8. Electrophoresis buffer, pH 8.7: Glycine, 2.8% w/v; SDS, 0.1% w/v; Tris base 0.58% w/v
9. Tracking dye: Bromphenol blue, in solution 5, 0.1% w/v

SDS was obtained from Serva and iodoacetamide from Sigma. Acrylamide (technical grade) and bisacrylamide were obtained from Eastman and used without recrystallization.

First SDS Electrophoresis

Approximately 1 mg of lyophilized or precipitated cross-linked protein is dissolved in 25–50 μl of solution 5 and incubated for 15 min at 65°. Tracking dye is added immediately before electrophoresis. The gels are poured in silicon-coated glass tubes 14 cm × 0.4 cm (i.d.). The separation gel (solution 7) and the upper gel (solution 6) are 10 cm and 1 cm, respectively. Electrophoresis toward the anode is at 2 mA/gel for 3.5 hr at room temperature using solution 8 as the electrolyte. In experiments for which it is desirable to have marker proteins, such as total 50 S protein, for the second electrophoresis (see Fig. 6), this is added to the origin of the gel 10 min prior to completion of electrophoresis. After electrophoresis, the gel is removed from the tube by smashing the glass and then soaked in 50 ml of solution 8 made 3% (v/v) in 2-mercaptoethanol, for 15 min at 65°. The gel is then incubated for 30 min at room temperature in another 50 ml of solution 8 in which the pH is adjusted to 6.8. The gel is now ready to be embedded as the origin of the second polyacrylamide gel, which is a slab.

Second SDS Electrophoresis

The apparatus is a modification of that described previously[9] and consists of two glass plates (24 cm × 12 cm) separated by Plexiglas spacers (0.4 cm) clamped to a Plexiglas unit with upper and lower reservoirs for electrolyte. The reduced gel from the first dimension is embedded at the origin of the gel slab by first squeezing it between the glass plates and pouring the separation gel on top of it. The composition of the gel is identical to that of the first electrophoresis. Tracking dye (solution 9) is applied just above the embedded gel cylinder prior to electrophoresis. Electrophoresis is carried out for 1 hr at 50 V followed by 30 hr at 90 V using solution 8 as the electrolyte. The gel slab is stained for 30 min in a solution containing methanol, glacial acetic acid, and water (5:1:5 by volume) with 0.55% (w/v) Amido black.

Purification of Radioactive, Cross-Linked Ribosomal Proteins prior to Diagonal Gel Electrophoresis

Figure 3 shows stained diagonal gels for ribosomal proteins from both the 30 S and 50 S subunits. The patterns are complex. Many protein dimers can be identified by the criteria mentioned previously: the additivity of apparent molecular weights ($\text{monomer}_a + \text{monomer}_b = \text{cross-linked species}_c$), and the finding of a and b on the same vertical line descending from c (see Figs. 2 and 3). Many more protein dimers are present than can be readily identified. This is because many ribosomal proteins have the same or similar molecular weights. Accordingly, procedures were developed to simplify the samples analyzed by diagonal gel electrophoresis. The cross-linked subunits are first extracted with increasing concentrations of LiCl.[9] Then each extracted fraction is separated by electrophoresis in polyacrylamide/urea gels.[10] The gel is sliced into 0.5-cm segments, each of which serves as one sample for diagonal gel electrophoresis.

Extraction with LiCl

Solutions

10. Solution 2 with 1.0 *M* LiCl
11. Solution 2 with 1.5 *M* LiCl
12. Solution 2 with 2.0 *M* LiCl

[9] G. A. Howard and R. R. Traut, this series, Vol. 30, p. 526.
[9] A. Sommer and R. R. Traut, *J. Mol. Biol.* 106, 995 (1976).
[10] U. C. Knopf, A. Sommer, J. Kenny, and R. R. Traut, *Mol. Biol. Rep.* 2, 35 (1975).

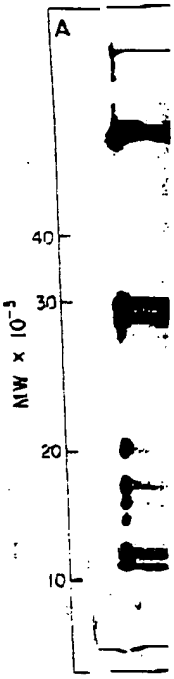


FIG. 3. Two-
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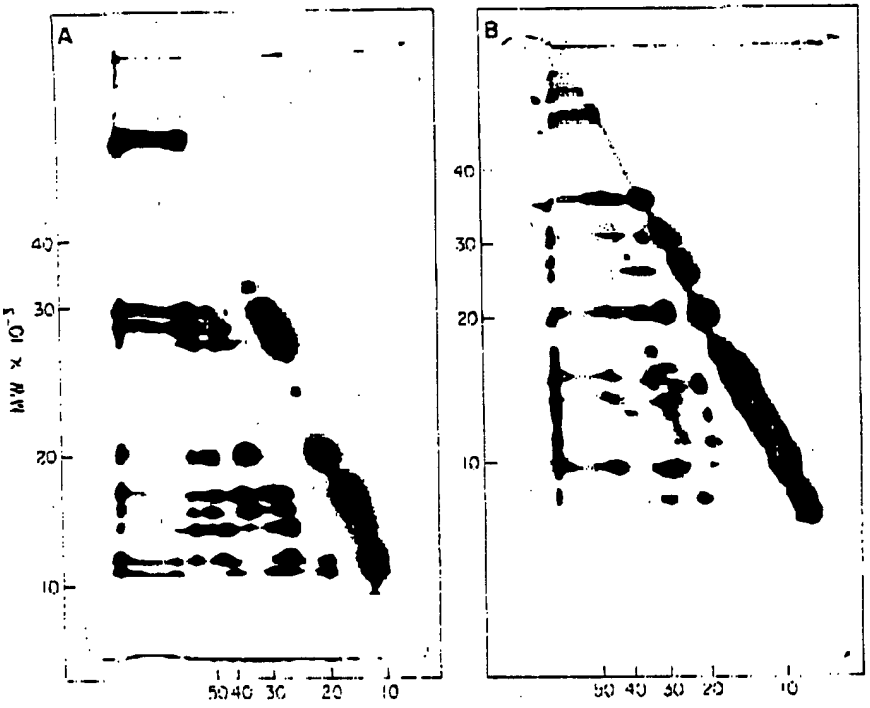

FIG. 3. Two-dimensional diagonal polyacrylamide/SDS gels of proteins extracted from ribosomal subunits modified with 2-iminothiolane and oxidized. (A) 30 S subunits. (B) 50 S subunits. Electrophoresis was as represented in Fig. 2 and described in the text, except that the acrylamide concentration for the 30 S subunits was 13.5%.

Cross-linked 50 S ribosomal subunits labeled *in vivo* with [^{35}S]sulfate[8] (180 A_{260} units or 4 mg of protein) in 4.2 ml of solution 2 with 80 m*M* iodoacetamide are adjusted to 0.2 m*M* EDTA and mixed with an equal volume of solution 10. The mixture is incubated for 5 hr at 4° and then centrifuged for 17 hr at 27,000 rpm in a Beckman SW 56 rotor. The radioactive supernatant fraction is mixed with 300 μg of nonradioactive, uncross-linked total 50 S ribosomal protein, dialyzed against 6% acetic acid, and lyophilized. The procedure is repeated on the pelleted protein-deficient ribosomal subunit fraction with solutions 11 and 12 successively to extract additional ribosomal protein fractions. At each step the protein-deficient "core" is first suspended in solution 2 containing 80 m*M* iodoacetamide and incubated for 30 min at 30° in order to alkylate any free

sulfhydryl groups that might become exposed by removal of proteins. Free sulfhydryl groups are capable of undergoing either random intermolecular oxidation or disulfide interchange when the proteins are extracted from the intact ribosomal subunit or core particle. The final core particle is also alkylated and then treated with 66% acetic acid, 33 m*M* $MgCl_2$ to extract the remaining protein and precipitate the RNA. The protein is dialyzed against 6% acetic acid and lyophilized. All protein fractions are enriched for specific cross-links as well as monomeric proteins. The recovery of protein and radioactivity is shown in Table I.

Electrophoretic Fractionation

Lyophilized protein fractions (see Table I for amounts) are resuspended in 50 μl of a buffer containing 8 *M* urea and 40 m*M* iodoacetamide. Pyronine G is added to the solution as a tracking dye. Samples are fractionated by electrophoresis in 4% polyacrylamide gels (10 cm × 0.4 cm i.d.) containing 6 *M* urea and 38 m*M* bis-Tris·acetate, pH 5.5.[10] Electrophoresis is carried out for 5 hr at 1 mA per gel toward the cathode. A detailed description of this gel system follows in the next section. The gel is removed from the glass tube and sliced into 20 equal fractions of 0.5 cm length. The gels are illustrated in Fig. 4, along with unfractionated cross-linked 50 S ribosomal protein and noncross-linked monomeric total 50 S protein.

Each 0.5-cm gel slice containing radioactive protein from cross-linked ribosomal subunits is inserted at one end of a 10.5 cm × 0.4 cm (ID) silicon-coated glass tube. The end containing the gel slice is covered with

TABLE I
EXTRACTION OF CROSS-LINKED ^{35}S-LABELED 50 S SUBUNITS WITH LiCl[a]

Protein fraction	Protein (mg)	Radioactivity (cpm × 10^{-6})	Percentage recovered
0.00–0.50 *M* LiCl	1.55	266	38.7
0.50–0.75 *M* LiCl	0.76	130	18.9
0.75–1.00 *M* LiCl	0.21	36	5.2
1.00 *M* LiCl core (extracted with acetic acid)	0.23	40	5.8

[a] The total protein present prior to initial extraction was 4 mg (12 mg of 50 S subunits) containing 687 × 10^6 cpm. Protein recovered was calculated from specific radioactivity as measured after dialysis against 6% acetic acid. The recovery was 68%.

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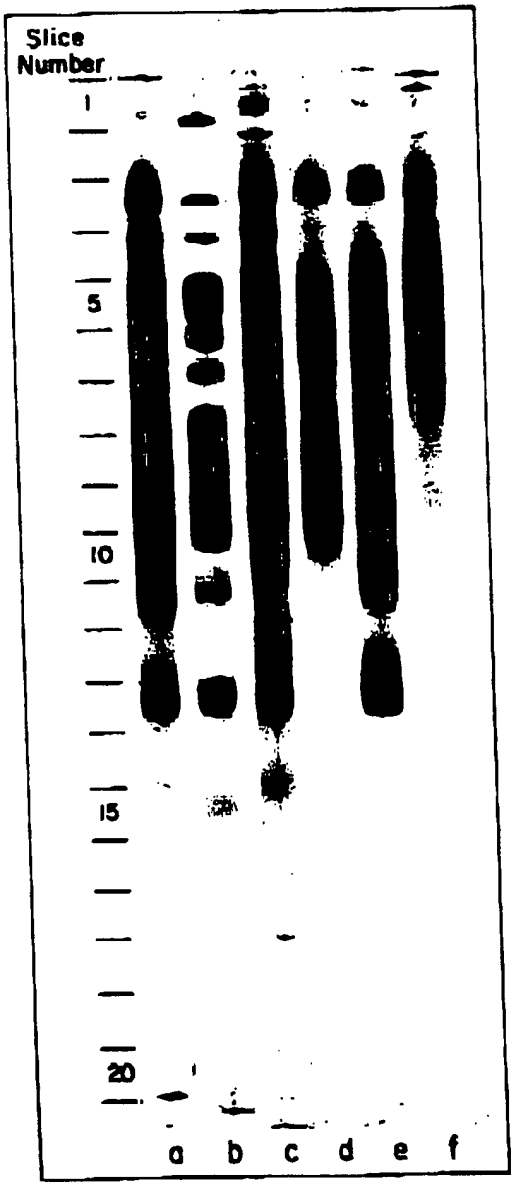


FIG. 4. Polyacrylamide/urea gel electrophoresis of protein fractions extracted from cross-linked 50 S ribosomal subunits at different concentrations of LiCl. The acrylamide concentration was 4% and the pH was 5.5. Details are given in the text. a, Cross-linked total 50 S protein; b, noncross-linked total 50 S protein; c–e, protein extracted from cross-linked 50 S subunits with LiCl: c, 0–0.5 *M*; d, 0.5–0.75 *M*; e, 0.75–1.0 *M*; f, proteins remaining after extraction with 1.0 *M* LiCl. The core particle was extracted with 66% acetic acid.

Parafilm. The tube is inverted and filled with a polyacrylamide/SDS gel solution containing 17.5% acrylamide (solution 7), and electrophoresed as previously described. The second electrophoresis is also carried out as previously described and is followed by staining the gel with Amido black. An example of a simplified diagonal gel pattern resulting from these fractionation steps is shown in Fig. 6A.

Two-Dimensional Polyacrylamide/Urea Gel Electrophoresis for the Identification of Radioactive Proteins from Diagonal Gels

The position of a stained protein spot on a diagonal gel is insufficient, in many cases, for unambiguous identification of ribosomal proteins since many have similar molecular weights. It is for this reason that radioactive protein is used throughout the methods described here. Radioactive proteins beneath the diagonal are eluted, mixed with total noncross-linked, nonradioactive 50 S ribosomal protein, and analyzed as described below.

Solutions and Acrylamide Gel Composition

Protein Elution

13. Tris-acetate, pH 7.8, 100 m*M*; SDS, 1% w/v; 2-mercaptoethanol, 1% v/v
14. Tris-acetate, pH 7.8, 50 m*M*; urea, 8.0 *M*; 2-mercaptoethanol, 1% v/v

First electrophoresis

15. Sample buffer: urea, 8.0 *M*; iodoacetamide, 40 m*M*
16. Upper gel, pH 4.7: acrylamide, 4% w/v; MBA, 0.066% w/v; urea, 6.0 *M*; Bis-Tris, 38 m*M*; TEMED, 0.02% v/v
17. Separation gel, as solution 16 except pH 5.5

The pH of solutions 16 and 17 is adjusted with glacial acetic acid after mixing the components. To catalyze polymerization of the gels, 5 ml per liter of ammonium persulfate (10% w/v) are added to the degassed gel solutions.

18. Electrophoresis buffers: (upper reservoir) Bis-Tris-acetate, pH 3.7, 20 m*M*; (lower reservoir) Bis-Tris-acetate, pH 7.0, 20 m*M*
19. Tracking dye: pyronine G, 0.5% w/v in solution 15

Second Electrophoresis

20. Separation gel, pH 4.6: acrylamide, 18% w/v; MBA, 0.25% w/v; urea, 6.0 *M*; glacial acetic acid, 920 m*M*; KOH, 48 m*M*; TEMED, 0.58% v/v

The second electrophoretic separation is a slight modification of the system of Kaltschmidt and Wittmann.[11] Polymerization is catalyzed by the addition of 30 ml per liter of ammonium persulfate (10% w/v).

21. Electrophoresis buffer, pH 4.0: glycine, 180 m*M*; acetic acid, 6 m*M*
22. Staining solution: trichloroacetic acid, 12.5% w/v; Coomassie Blue G-250, 0.0125% w/v

Urea "ultra pure" and Bis-Tris were purchased from Sigma, glycine from Eastman, trichloroacetic acid (analytical grade) from Mallinckrodt, and Coomassie Blue G-250 from Pierce.

Elution of Proteins from Diagonal Gels

The stained spots from diagonal gels are cut out. The gel is then macerated with a glass rod to make a fine slurry suspended in an adequate volume (200–500 μl depending on size of gel segment) of solution 13. The slurry is incubated for 15 min at 65° and then cooled to room temperature. Nonradioactive, noncross-linked total 50 S protein, 300 μg, is added. This carrier is added in order to decrease loss of radioactive protein and to act as marker on subsequent two-dimensional polyacrylamide/urea gel electrophoresis. The mixture of protein and gel particles is adjusted to approximately 8 *M* urea by adding solid crystals, approximately doubling the volume, and applied to a column (2.5 cm × 0.8 cm for a 1.0 ml sample) containing Bio-Rad Dowex 1-X8 (20–50 mesh, acetate form) equilibrated with solution 14 to remove SDS. The sample enters the column under gravity and is washed through with 0.5 ml of 66% acetic acid. SDS and stain are bound to the resin. The eluate is dialyzed against 6% acetic acid and lyophilized.

First Electrophoresis

Each sample is resuspended in 25–50 μl of solution 15, mixed with tracking dye (solution 19), and applied to silicon-coated glass tubes (12 cm × 0.3 cm i.d.) containing a separation gel (solution 17) and an upper gel (solution 16), which are 10 cm and 0.5 cm, respectively. The acrylamide concentration is low (4%), and the separation is predominantly due to differences in charge. Electrophoresis is carried out for 5 hr at 1 mA/gel at room temperature toward the cathode. The pH of the upper electrophoresis buffer is 3.7 and that of the lower, pH 7.0. The low pH of the

[11] E. Kaltschmidt and H. G. Wittmann, *Anal. Biochem.* **36**, 401 (1970).

upper buffer allows the entry of all ribosomal proteins including the acidic proteins L7 and L12 into the gel.

Second Electrophoresis

After completion of the first electrophoresis, the gel is removed from the tube and embedded at the origin of the second urea gel slab. The high acrylamide concentration (18%) results in a separation of the proteins based predominantly on size. The apparatus used is similar to that described earlier.[8] The dimensions of the gel are 10 cm × 12 cm × 0.3 cm. After polymerization of the gel (solution 20) and application of tracking dye (solution 19), electrophoresis is carried out toward the cathode for between 7 and 16 hr at 150 V to 65 V in a glycine buffer at pH 4.0 (solution 21). The gel slabs are stained for 30 min in 100 ml of solution 22 containing Coomassie Blue G-250.[12] The stained protein spots are intensified by soaking the gel in 100 ml of 6% acetic acid for 30–60 min. To clear the gel of trichloroacetic acid, which is required if the gel is to be dried in preparation for radioautography, the gel is transferred to another 100 ml of 6% acetic acid and slowly shaken for approximately 18 hr. The gel is then dried onto Whatman 3 MM paper under vacuum with heating using a commercially available gel slab dryer unit.

Identification of Individual Ribosomal Proteins by Radioautography

The gel is exposed to X-ray film (Kodak No-Screen medical X-ray film) by placing the gel directly against the film and clamping between two 1 cm-thick foam pads and two 15 cm × 20 cm × 0.7 cm plywood sheets. The exposure time depends on the radioactivity of the sample; for example, 10,000 cpm of ^{35}S-labeled protein requires an exposure time of approximately 2 weeks. The X-ray film is developed using standard procedures.

Results and Discussion

Figure 3 shows diagonal gels of both cross-linked 30 S and 50 S ribosomal subunits of *Escherichia coli*. The complexity of the patterns beneath the diagonal is apparent. An exhaustive analysis of such patterns is made difficult because of overlap of spots due to cross-linking among proteins, many of which have the same or similar molecular weights. The specificity of the patterns is notable. The patterns, though difficult to analyze in detail, are characteristic "fingerprints" of the protein topog-

[12] W. Diezel, G. Kopperschlager, and E. Hofmann, *Anal. Biochem.* **48**, 617 (1972).

raphy of each subunit. There are differences in position and intensities of spots: some proteins are frequently cross-linked and often to more than one neighboring protein; others are less frequently found in cross-links. Partial purification of the mixture of the proteins extracted from cross-linked 50 S ribosomal subunits was obtained by salt extraction and electrophoresis. The gels in Fig. 4 illustrate the fractionation achieved by extraction of the particle with increasing concentrations of LiCl. The horizontal lines indicate the 0.5-cm slices that were used for diagonal gel electrophoresis.

The polyacrylamide/urea gel electrophoresis system used for the final identification of ribosomal proteins eluted from diagonal gels is illustrated in Fig. 5. In this example total 70 S ribosomal protein was analyzed. However, it is clear that the system separates as discrete spots all the 50 S proteins. Thus the elution of a radioactive component from a diagonal gel, mixing with nonradioactive total 50 S protein, and electrophoresis in this system followed by staining and radioautography, leads to its unambiguous identification.

A diagonal gel of one of the purified fractions from cross-linked 50 S ribosomal subunits is shown in Fig. 6A. Comparison with Fig. 3B shows the degree of purification obtained. Two pairs of spots are indicated by arrows. A_1 and A_2 fall on the same vertical line whose intercept on the horizontal axis indicates a molecular weight for the cross-linked species of 31,000. The molecular weights of A_1 and A_2 given by the intercepts on the vertical axis are 20,800 and 10,100, respectively, giving a sum equal to that of the putative cross-linked dimer. Component A could be L5 and/or L6, judged from its mobility in SDS gels, and A_2 could be one of four different proteins. The radioactive components were eluted and analyzed by electrophoresis in the two-dimensional polyacrylamide/urea gel system. Figure 6B shows a radioautograph of the gel. The dark spots correspond to L5 and L25 as determined by superposition of the X-ray film on the stained gel. Similar analysis of B_1 and B_2 show them to have molecular weights consistent with their presence in a cross-linked dimer and their identity as L17 and L32. More than thirty protein dimers have been identified from the 50 S ribosomal subunit using the methods described here. While the purification procedures are time consuming, they simplify greatly the identification of components of dimers. Purification also facilitates identification of dimers formed in moderate to low yield. Important in this regard is the fact that all protein pairs identified using the purified fractions appear on diagonal gels like that shown in Fig. 3B of the total cross-linked protein.

There are many possible explanations for the variability in the yield of cross-linked protein pairs: differences in reactivity of lysine residues

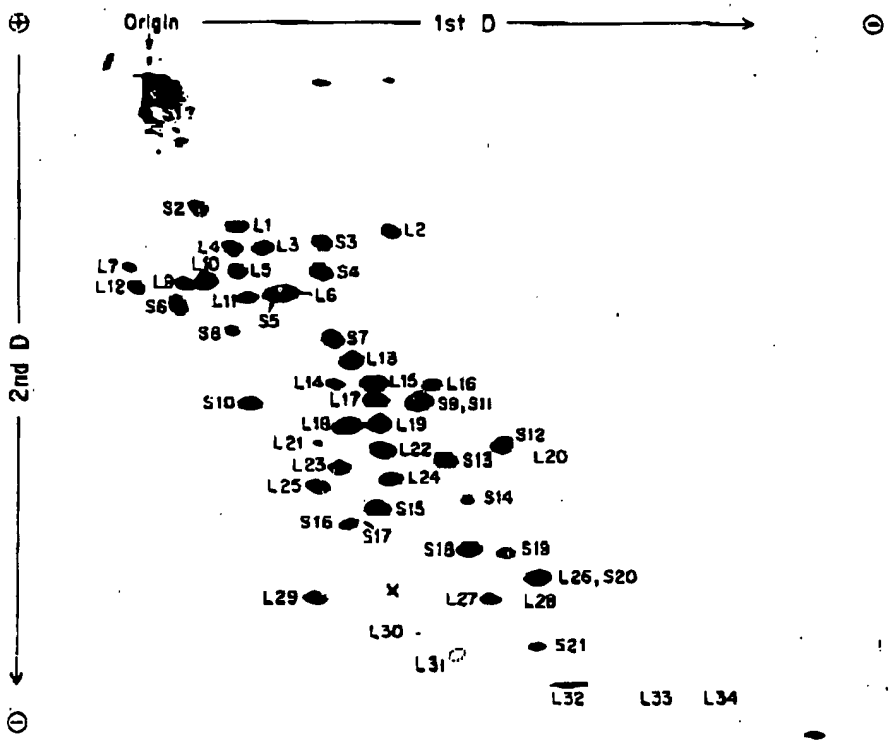
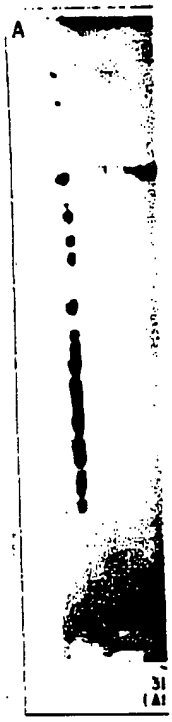


FIG. 5. Two-dimensional polyacrylamide/urea gel electrophoresis of total 70 S ribosomal protein of *Escherichia coli*. Protein was extracted from 70 S ribosomes with 66% acetic acid, dialyzed, and lyophilized. Electrophoresis was as described in Fig. 4 for the first electrophoresis and at pH 4.6 in 18% acrylamide for the second electrophoresis. See text for details. The numbering scheme conforms to that of E. Kaltschmidt and H. G. Wittmann, *Proc. Natl. Acad. Sci. U.S.A.* 67, 1276 (1970). The system clearly resolves all proteins except S16/S17 and S9/S11. L26 and S20 are the same protein as that found on both subunits. The spot marked X is a 50 S protein not previously reported.

and of the sulfhydryl groups introduced; the relative "isolation" of certain proteins, or lysines contained therein, from other proteins, possibly due to shielding by RNA; possible compositional and conformation heterogeneity in the population of ribosomal subunits; competition between dimer formation and the formation of higher cross-linked oligomers; and, perhaps most important, competition between intermolecular cross-linking and intramolecular disulfide bond formation.[3,4] It has been contended that disulfide cross-linking leads to artifacts: that cross-linked protein dimers may be due to disulfide interchange and/or random oxidation and



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[13] H. Pertz
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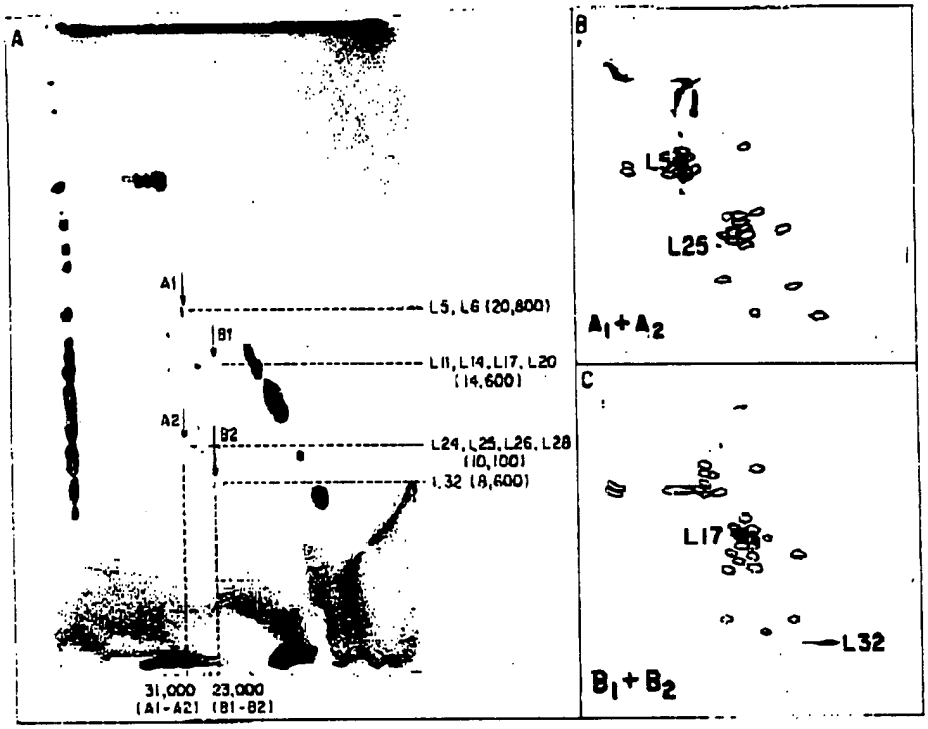

FIG. 6. Two-dimensional diagonal polyacrylamide/SDS gel of a purified fraction from cross-linked 50 S subunits, and radioautographs of two-dimensional polyacrylamide/urea gels illustrating identification of cross-linked monomeric proteins. Slice number 6 from panel f (Fig. 4) was analyzed by diagonal gel electrophoresis (A). The radioactive proteins A1, A2, B1, and B2 were eluted from the gel, mixed with nonradioactive total 50 S protein, and electrophoresed in the two dimensional polyacrylamide/urea gel system shown in Fig. 5. (B) and (C) are radioautographs in which the dark spots represent the radioactive components and the dotted circles represent stained marker proteins whose positions were used to identify the proteins of interest.

may not reflect protein neighborhoods existing in the intact ribosomal subunit.[13,14] The methods described here incorporate deliberate procedures to preclude such possible artifacts. Alkylation with iodoacetamide is included at each step of the procedure to minimize or exclude the presence of free sulfhydryl groups, necessary for either interchange or

[13] H. Peretz, H. Towbin, and D. Elson, *Eur. J. Biochem.* 63, 83 (1976).
[14] C. G. Kurland, *in* "Molecular Mechanisms of Protein Biosynthesis" (H. Weissbach and S. Pestka, eds.), p. 81. Academic Press, New York, 1977.

nonspecific oxidation. The results, however, are nearly identical to those obtained earlier[5,7] when acidification alone was employed to prevent the possible occurrence of nonspecific events. The cross-links reflect reactions which take place in the intact ribosomal subunit. The procedures do not detectably alter the structure of the subunit.

Other cleavable cross-linking reagents have been employed in the study of ribosome protein topography and in other systems. They include dimethyl 3,3'-dithiobispropionimidate,[15,16] dithiobis(succinimidyl)propionimidate,[17] tartryl diazides,[18] and, *N,N'*-bis(2-carboxyimidoethyl) tartarimide.[19] Diagonal gel electrophoresis has also been employed by others, both with the disulfide reagents and the tartaric acid derivatives. A method for cleaving cross-links formed with dimethyl suberimidate has been reported.[20] A method quite similar to that described here has been used in an investigation of chromatin structure employing the reagent methyl 3-mercaptopropionimidate.[21]

The use of 2-iminothiolane to form interprotein cross-links has several distinct advantages: the disulfide cross-link is readily reversed by reduction; the compound is relatively stable in solution compared to other imidates, probably owing to its five-membered ring structure; it is available commercially; the cross-linking reaction is separated from the initial protein modification reaction with the consequence that any lysine that reacts with the reagent is a potential site for cross-linking. Other imidate cross-linking reagents are bifunctional imidates; e.g., dithiobispropionimidate, dimethylsuberimidate and may give a lower yield of cross-linking due to the likelihood that one functional group may react with protein while the second may hydrolyze prior to reaction with a second protein.

Protein cross-linking with 2-iminothiolane and oxidation to form disulfide linkages between neighboring proteins and analysis by diagonal gel electrophoresis are general techniques. Detailed methods have been described here as they have been applied to the investigation of the protein topography of ribosomes. However, with slight modifications the methods for cross-linking and for diagonal gel electrophoresis can be readily applied to the investigation of other protein complexes.

[15] K. Wang, and F. M. Richards, *J. Biol. Chem.* 249, 8005 (1974).
[16] A. Ruoho, P. A. Bartlett, A. Dutton, and S. J. Singer, *Biochem. Biophys. Res. Commun.* 63, 417 (1975).
[17] A. J. Lomant and G. Fairbanks, *J. Mol. Biol.* 104, 243 (1976).
[18] L. C. Lutter, C. G. Kurland, and G. Stöffler, *FEBS Lett.* 54, 144 (1975).
[19] J. R. Coggins, J. Lumsden, and A. D. B. Malcolm, *Biochemistry* 16, 1111 (1977).
[20] A. Expert-Bezançon, D. Barritault, M. Milet, M.-F. Guérin, and D. H. Hayes, *J. Mol. Biol.* 112, 603 (1977).
[21] J. O. Thomas and R. D. Kornberg, *FEBS Lett.* 58, 353 (1975).

3-3 The antibody molecule can readily be cleaved into functionally distinct fragments.

The antibody molecule comprises three equal-sized globular portions joined by a flexible stretch of polypeptide chain known as the **hinge region** to form a crude 'Y' shape (see Fig. 3.1). Each arm of the Y is formed by the association of a light chain with the amino-terminal half of a heavy chain, whereas the trunk of the Y is formed by the pairing of the carboxy-terminal halves of the two heavy chains. The association of the heavy and light chains is such that the V_H and V_L domains are paired, as are the C_H1 and C_L domains. The C_H3 domains pair with each other but the C_H2 domains do not interact; carbohydrate side chains attached to the C_H2 domains lie between the two heavy chains. The two antigen-binding sites are formed by the paired V_H and V_L domains at the end of the two arms of the Y (see Fig. 3.1, center panel).

Proteolytic enzymes (proteases) that cleave polypeptide sequences have been used to dissect the structure of antibody molecules and to determine which parts of the molecule are responsible for its various functions. Limited digestion with the protease papain cleaves antibody molecules into three fragments (Fig. 3.4). Two fragments are identical and contain the antigen-

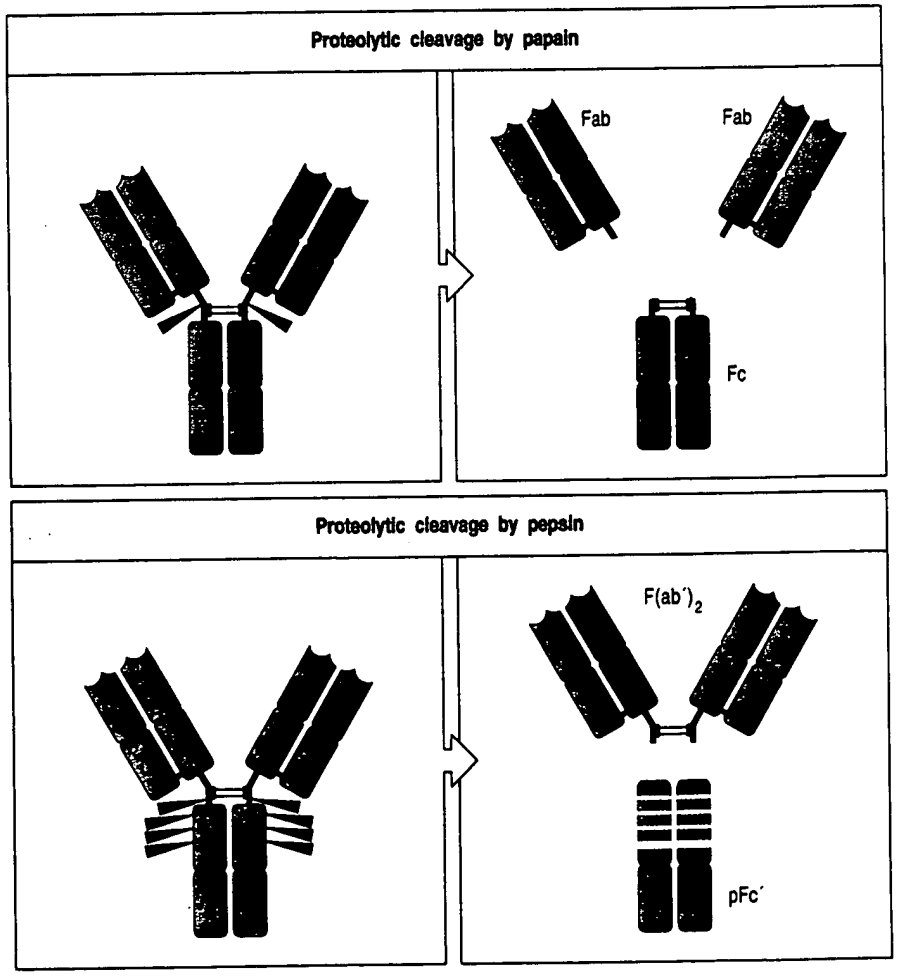

Fig. 3.4 The Y-shaped immunoglobulin molecule can be dissected by partial digestion with proteases. Papain cleaves the immunoglobulin molecule into three pieces, two Fab fragments and one Fc fragment (upper panels). The Fab binds antigens. The Fc is crystallizable and contains C regions. Pepsin cleaves an immuno-globulin to yield one $F(ab')_2$ fragment and many small pieces of the Fc fragment, the largest of which is called the pFc' fragment (lower panels). $F(ab')_2$ is written with a prime because it contains a few more amino acids than Fab, including the cysteines that are necessary for the disulfide bonds.

binding activity, and these are termed the **Fab fragments**, for Fragment antigen binding. The Fab fragments correspond to the arms of the antibody molecule, which contain the complete light chains paired with the V_H and C_H1 domains of the heavy chains. The other fragment contains no antigen-binding activity but was originally observed to crystallize readily, and for this reason was named the **Fc fragment**, for Fragment crystallizable. This fragment corresponds to the paired C_H2 and C_H3 domains and is the part of the antibody molecule that interacts with effector molecules and cells.

The exact pattern of fragments obtained after proteolysis depends on where the protease cleaves the antibody molecule in relation to the disulfide bonds that link the two heavy chains. These lie in the hinge region between the C_H1 and C_H2 domains, and, as illustrated in Fig. 3.4, papain cleaves the antibody molecule on the amino-terminal side of the disulfide bonds, releasing the two arms of the antibody as separate Fab fragments, whereas in the Fc fragment the carboxy-terminal halves of the heavy chains remain linked.

A second protease, pepsin, cleaves in the same general region of the antibody molecule as papain but on the carboxy-terminal side of the disulfide bonds (see Fig. 3.4), producing a fragment, the $F(ab')_2$ fragment, in which the two arms of the antibody molecule remain linked. In this case the remaining part of the heavy chain is cut into several small fragments. The $F(ab')_2$ fragment has exactly the same antigen-binding characteristics as the original antibody but is unable to interact with any effector molecule and thus is of potential value in therapeutic applications of antibodies as well as in research into the role of the Fc portion.

Genetic engineering techniques also now permit the construction of a truncated Fab comprising only the V region of a heavy chain linked by a stretch of synthetic peptide to a V region of a light chain. This is called **single-chain Fv**, named from Fragment variable. Fv molecules may become valuable therapeutic agents because of their small size, allowing ready tissue penetration. They may be coupled to protein toxins to yield immunotoxins with potential application, for example, in tumor therapy.

3-4 The immunoglobulin molecule is flexible, especially at the hinge region.

The hinge region that links the Fc and Fab portions of the antibody molecule is in reality a flexible tether, allowing independent movement of the two Fab arms, rather than a rigid hinge. For example, electron microscopy of antibody complexes with a bivalent hapten capable of crosslinking two antigen-binding sites demonstrates that the angle between the two Fab arms can vary (Fig. 3.5). Some flexibility is also found at the junction between the V and C domains, allowing bending and rotation of the V domain relative to the C domain—for example, in the crystal structure of the antibody molecule shown in Fig. 3.1 (top panel), not only are the two hinge regions clearly different, but the angle between the V and C domains in each of the two Fab arms is also different. This range of motion has led to the junction between the V and C domains being referred to as a 'molecular ball-and-socket joint'. Flexibility at both the hinge and V–C junction enables the binding of both arms of the antibody molecule to sites that are different distances apart, for instance sites on bacterial cell-wall polysaccharides. Flexibility of the hinge also permits the interaction of antibodies with the antibody-binding proteins that mediate immune effector mechanisms, as will be described in Chapter 9.

IMMUNO BIOLOGY

THE IMMUNE SYSTEM IN HEALTH AND DISEASE

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Charles A. Janeway, Jr.
Yale University Medical School

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are lost. To examine the incorporation into these proteins, "three-dimensional" electrophoresis of acetic acid-soluble protein is available and is described in this volume [41].[18]

It must be added that these methods are useful for study of the metabolism of ribosomal proteins *in vivo*, as shown by our previous reports.[4-7]

[18] Y. Nabeshima, K. Imai, and K. Ogata, to be published.

[43] Cross-Linking of Ribosomes Using 2-Iminothiolane (Methyl 4-Mercaptobutyrimidate) and Identification of Cross-Linked Proteins by Diagonal Polyacrylamide/Sodium Dodecyl Sulfate Gel Electrophoresis[1]

By JAMES W. KENNY, JOHN M. LAMBERT, and ROBERT R. TRAUT

Many biological structures contain assemblies of different proteins. It is frequently valuable to determine the spatial relationships among the different protein components of the multiprotein complex. Bifunctional reagents have been used effectively to cross-link one protein component to others that occupy a suitably "neighboring" site in the structure or complex under investigation. A problem frequently encountered is that of identification of the monomeric components of cross-linked dimers or oligomers. The presence of a readily cleavable bond in the cross-linking reagent permits reversal of the cross-linking reaction and regeneration of monomeric components from isolated cross-linked complexes, thus facilitating their identification. Methods are described here that employ reversible cross-linking and analysis of a complex mixture of cross-linked products. They have been used successfully in the investigation of the protein topography of ribosomal subunits of *Escherichia coli*. They are of general applicability and are useful in the investigation of many other biological structures containing multiple protein components.

The reagent 2-iminothiolane, formerly called methyl 4-mercaptobutyrimidate[2,3] reacts with lysine amino groups in the intact ribosomal subunit to form amidine derivatives containing sulfhydryl groups. Disulfide bonds form when the modified subunit is subjected to oxidation.

[1] Supported by a research grant from the U.S. Public Health Service (GM 17924).
[2] R. R. Traut, A. Bollen, T. T. Sun, J. W. B. Hershey, J. Sundberg, and L. R. Pierce, *Biochemistry* 12, 3266 (1973).
[3] R. Jue, J. H. Lambert, L. R. Pierce and R. R. Traut, *Biochemistry*, in press (1978).

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